Is Refrigeration Enough to Restrain Foodborne Pathogens?

ABSTRACT

Holding foods at 5°C has traditionally been viewed as adequate to restrain the growth of foodborne pathogens. However, a group of "new" foodborne pathogens has emerged, some of which are capable of competitive growth at 5°C in foods. Bacteria fitting this criterion include *Clostridium botulinum* type E, *Yersinia enterocolitica,* enterotoxigenic *Escherichia coli, Listeria monocytogenes* and *Aeromonas hydrophilia*. A second area discussed is the effect of low temperature (5°C) on survival of foodborne pathogens. Both *Campylobacter jejuni* and *Brucella* survive for longer periods at 5°C compared to 25 or 37°C. A third area considered is the growth of certain pathogens *(Salmonella, Staphylococcus aureus, Vibrio parahaemolyticus, Bacillus cereus)* at temperatures slightly above 5°C up to 12°C. Hence, temperature abuse of a food could readily generate a hazard in a food. The use of refrigeration (5°C holding of a food) can no longer be deemed sufficient to keep foods safe from bacterial hazards either by growth of the "new" pathogens or increased survival. Further, even brief temperature abuse can create hazards from certain bacteria.

With the advent of mechanical refrigeration, food processors and consumers were able to increase the microbiological shelf life of foods. The widespread use of mechanical refrigeration permitted fresh foods to become available to wider geographical areas and to be available for longer periods. Food products which have benefitted from the use of mechanical refrigeration included fresh and processed meats and dairy products, poultry, and fresh fruit and vegetables. In recent years, the list has grown to include an ever increasing array of items.

Mechanical refrigeration (MR), a system to maintain the temperature of a food at 5°C (41°F) preserves food by slowing down or eliminating the production of various undesirable microbial end-products of spoilage organisms, and preventing growth and/or toxin production of pathogenic organisms. In addition, MR slows down the autodegration of food. By the mid-1950s, it became evident that MR was not adequate to prevent completely the growth of food spoilage organisms. At the time, the growth of various spoilage bacteria, yeasts and mold was observed to occur in a wide range of meat, fish, poultry and dairy products held at 5°C *(4,25,69,78,79)*.

Initially, microorganisms which could spoil food held at 5°C were termed psychrophiles. Based on the etiology of the term, these would be cold-loving species that should have a temperature optimum of 5°C. However, most of the bacteria which grow in food held at 5°C grow optimally at 25°C, and are often capable of growth at 30°C. Hence, they are not true psychrophiles. Based on these considerations, Eddy *(24)* proposed the term psychrotroph to describe any microorganism capable of growth at 5°C, regardless of its temperature optimum. A further discussion of the terms psychrophile and psychrotroph as they apply to organisms and growth data already in the literature is provided by Ingram *(43)*. The term psychrotroph has found widespread usage in the current food microbiology literature when describing spoilage organisms growing during refrigerated storage of food.

The growth, physiology and metabolism of psychrotrophic bacteria have been reviewed by various authors *(27,42,55,63,83)*. The exact mechanism(s) which enables certain strains of microorganisms to grow at 5°C, while closely related strains cannot, is not known although many comparative studies have been done.

Although it was determined that MR only retards the spoilage of food products, MR was still considered adequate to prevent growth and/or toxin formation by foodborne pathogens, or at least the foodborne pathogens known in the mid-1950s. Hence, it was thought that any food held at 5°C or below should be "pathogen-free" and safe. However, in the early 1960s, type E *Clostridium botulinum* was shown to be the causative agent in outbreaks of food poisoning from fish products. This organism was subsequently shown to grow and produce toxin at 38°F (3.3°C) in a beef stew medium. Since that time, other bacteria have been found to grow and/or produce toxins at 5°C or less in foods. The primary purpose of this discussion is to review the growth and/or toxin formation by bacteria capable of growth at 5°C. Included in this list of psychrotrophic foodborne pathogens are: *C. botulinum* type E, *Yersinia enterocolitica,* enterotoxigenic *Escherichia coli, Listeria monocytogenes,* and *Aeromonas hydrophila*. The influence of temperature on the survival of certain bacteria will also be discussed because storage of contaminated food at 5°C (non-growing conditions) has

been shown to maintain the viability of certain bacterial strains. Additionally, this review will also consider briefly *Salmonella, Staphylococcus aureus, Bacillus cereus* and *Vibrio parahaemolyticus,* which are capable of growth at temperatures between 5 and 12°C, conditions approximating mild temperature abuse conditions for some foods. This last area is of particular concern in that various foods are often held above the ideal temperature of 5°C. Wyatt and Guy *(84)* reported that the temperatures in 7 of 10 retail meat-holding display cases surveyed were above 45°F (7.2°C). Torrey and Marth *(81)* observed that the air temperatures of home refrigerators ranged from 1.7 to 20.2°C. Bryan et al. *(15)* surveyed food-holding practices of airline catering operations; they found that many prepared foods were exposed to temperatures above 45°F (7.2°C) for several hours and that some of the equipment was incapable of maintaining food temperatures below 45°F (7.2°C).

CLOSTRIDIUM BOTULINUM TYPE E

Human botulism is caused principally by types A, B or E toxins *(65)*. Until the early 1960s, types A and B were the botulinal toxins predominantly associated with human food poisoning. However, more recently, there were outbreaks of botulism caused by type E toxin and these were associated with fish and marine products. Before that time, normal refrigeration (5°C) was considered adequate to prevent the hazard from botulism *(17,62)*. These outbreaks prompted a renewed interest in botulism, especially the possibility of growth and toxin production at low temperatures. In addition to its growth at low temperatures, *C. botulinum* type E is also unique in its distribution. In contrast to *C. botulinum* types A and B which are primarily soil organisms, *C. botulinum* type E is a marine (aquatic) organism *(16,40,49,62,73)*.

Several studies have been done to support the premise that normal refrigeration alone is not adequate to suppress the growth of type E *C. botulinum*. Schmidt et al. *(68)* reported that *C. botulinum* type E grew and produced toxin in heat-sterilized beef stew substrate at 38°F (3.3°C) within 31 d. Cann et al. *(17)* observed growth and toxin production by type E *C. botulinum* in fresh herring after 15 d storage at 5°C; the starting count of the organism was 10^2/vacuum package. They also observed that type E spores survived irradiation of the fish at 0.3 Mrads from a Co^{60} source and produced toxin more rapidly than in non-irradiated fish.

Growth and toxin production by *C. botulinum* type E has been observed at temperatures slightly above the target refrigeration temperature of 5°C. Kautter *(45)* observed growth and toxin production in inoculated, vacuum-packaged smoked fish held 5 d at 10°C. It is important to note that the toxic packages of fish did not show any signs of reduced quality. Solomon et al. *(74)* reported toxin production in crabmeat held at 12°C for 14 d. Cann et al. *(18)* observed growth and toxin production in vacuum-packaged irradiated (0.3 Mrads) herring, cod and haddock held at 10°C. As with their previous study, more toxin was produced in the irradiated samples.

In addition to the temperature aspects, a second important consideration for toxin production appears to be background flora. Cann et al. *(17,18)* found more toxin in irradiated fish. The spores survive irradiation, but the background flora was decreased. Kautter *(45)* also observed that, in open packages of smoked non-irradiated fish, surface spoilage developed rapidly and no toxin was formed, even at 31 d at 5°C (irradiated samples were toxic). Which specific component of the normal flora of fish is responsible for suppression is not known, but it is known that the presence of lactic acid bacteria plus a fermentable carbohydrate can provide protection from botulism in cured meat products *(61)*.

Simunovic et al. *(71)* have grouped nonproteolytic strains of *C. botulinum* types B and F with all strains of type E and indicated that all of these nonproteolytic organisms are capable of low temperature (5°C) growth. Hence, three nonproteolytic types of *C. botulinum* are now considered of potential concern for refrigerated foods.

YERSINIA ENTEROCOLITICA

Yersinia enterocolitica is a facultatively anaerobic, gram-negative, short rod-shaped bacterium currently classified as part of the family *Enterobacteriaceae (21)*. Bacteria with biochemical properties close to *Y. enterocolitica* have been isolated from various sources and these were originally designated as *Y. enterocolitica*-like organisms *(85)*. Bercovier and Mollaret *(8)* now classify these *Y. enterocolitica*-like bacteria as three separate species: *Y. intermedia, Y. frederiksonü* and *Y.kristensenii*. Biochemical characteristics differentiate these four species of *Yersinia (8,85)*.

In addition to their biochemical properties, *Y. enterocolitica* can be serotyped *(85)*, providing an excellent tool for tracing sources of the organism in various epidemiological studies. Other aspects of *Y. enterocolitica,* their growth and physiology, and occurrence in foods have been reviewed recently *(48,75,77,85)*.

Y. enterocolitica and related organisms can be isolated from a variety of foods, especially those of animal origin *(48,75)*. These same foods have been incriminated in several outbreaks of food poisoning *(10,48,70,75)*. Chocolate milk was the vehicle in the first major outbreak. In this outbreak *(10)*, the milk apparently became contaminated during processing after the pasteurization step. Several of the ill school children underwent appendectomies before the bacterial nature of the illness was determined. *Y. enterocolitica* food poisoning appears unique among food poisoning bacteria in that symptoms differ for various age groups *(82)*. Cases of gastroenteritis, mesenteric lymphadenitis and pseudoappendicitis predominate in childhood and adolescence, whereas cases of acute abdominal disorders, diarrhea and arthritis primarily occur in adults, with erythema nodosum the most striking

symptom in the older age groups. The children in the chocolate milk outbreak had mesenteric lymphadenitis and ileitis, which mimic acute appendicitis. In another outbreak, reconstituted powdered milk and turkey chow mein were the vehicles of transmission *(70)*. Appendectomies were performed on five out of the seven hospitalized patients.

Y. enterocolitica, as well as all members of the genus *Yersinia,* are capable of growth at 5°C *(8)*. In fact, one method of isolating *Y. enterocolitica* from food and clinical specimens involves an enrichment in alkaline phosphate buffer (pH 7.6) for 14 to 21 d at 4°C *(85)*. The high pH combined with the low temperature selects for *Y. enterocolitica* over the competing microflora. Aulisio et al. *(3)* have reported that the cold enrichment procedure is very useful in recovering *Y. enterocolitica* from a variety of meats, shellfish and vegetables. Schiemann *(66)* reported that the following procedure worked for inoculated beef stew: non-selective enrichment in Trypticase soy broth held at 22°C for 1 d followed by 4 to 7 d at 2 to 4°C and isolation on cefsulodin-irgasan-novobiocin (CIN) selective agar. Head et al. *(36)* also found that CIN agar was highly selective and almost completely inhibited background flora while it supported good growth of *Y. enterocolitica*.

Pathogens are often considered to be poor competitors when inoculated into food products. However, *Y. enterocolitica* can grow readily in raw beef and pork held at 1 to 7°C in the presence of the normal meat microflora *(33)*. In contrast, *Y. enterocolitica* was not able to grow competitively in milk *(76)*. Whether this represents differences in competitive microflora, nutritient content or some other factor(s) is not known.

Concerns over the apparently wide-spread occurrence of *Y. enterocolitica* and *Y. enterocolitica*-like organisms are not easily resolved and much additional work is needed. Hill et al. *(39)* indicated that only *Y. enterocolitica* of Nilehn's biotypes 2, 3 and 4 possess virulence factors associated with pathogenicity. Pathogenic significance has been ascribed to several characteristics observed in various isolates: the invasion of HeLa cells, tissue invasiveness as revealed by the Sereny test in guinea pigs or mice, lethality in adult or suckling mice, heat-stable enterotoxin elaboration, lethality in adult gerbils and detachment of monolayers of HEp-2 cells in tissue culture *(39)*. Except for the production of heat-stable enterotoxin and the invasion of HeLa cells, the virulence determinants of *Y. enterocolitica* are coded for by plasmids of 42 to 48 Mdal *(39)*. Hill et al. *(39)* have developed a procedure for detecting and enumerating *Y. enterocolitica* in foods by DNA colony hydridization techniques similar to the procedure developed for virulence plasmids in *Escherichia coli (37,38)*. Their procedure uses 26°C for incubation because higher temperatures (35 to 37°C) can cause loss of the virulence plasmid. Hence, enrichment and isolation at 26°C or below is important for retaining virulence factors of the organism.

LISTERIA MONOCYTOGENES

Listeria monocytogenes is a small coccoid to rod-shaped, gram-positive, motile bacterium. Because of its unique morphology, the organism is often designated as a coryneform contaminant during the workup of various clinical specimens. In contrast to common food poisoning organisms which generally cause a variety of gastrointestinal symptoms, *L. monocytogenes* can cause various, often very severe disorders: meningo-encephalitis, flu-like low grade septicemia *in gravida,* septicemia in the perinatal period, infectious mononucleosis-like syndrome, septicemia in adults (often imposed on other disorders), pneumonia, endocarditis, urethritis and abortion *(32)*. The organism itself is the cause of the symptoms. The suggested procedure for isolation of this organism involves cold (5°C) enrichment of the suspected material *(32)*. In certain specimens, the organism can only be isolated after cold enrichment. The organism is capable of growth at 4 to 6°C *(7,46)* in milk and lamb. Gray and Killinger *(32)* reported that growth in culture occurs from 3°C to about 45°C, with the optimum between 30 and 37°C.

Food has been a vehicle of *L. monocytogenes* in recent outbreaks of listeriosis. In the first *(67)*, a food preference survey implicated coleslaw as the vehicle of transmission. *L. monocytogenes* of the same serotype (4b) as the organism isolated from a patient's blood was isolated from a sample of coleslaw from his refrigerator. The coleslaw was prepared by a regional processor who had obtained the cabbage from a farm known to have had cases of ovine listeriosis. The farmer grew the cabbage in fields fertilized with both raw and composted manure from the sheep flock. After harvesting, the cabbage was stored on the farm and then shipped during the following winter and spring. The prolonged cold storage of the cabbage could have enriched for *L. monocytogenes* as well as caused a die-off of the normal cabbage microflora. Additionally, *L. monocytogenes* serotype 4b was isolated from two unopened packages of coleslaw purchased in two different retail markets and held for a prolonged period of cold enrichment.

In the second outbreak, pasteurized whole and 2% milk from a food chain was the vehicle of transmission *(28)*. There were 49 cases of listeriosis with 14 deaths. The patients were hospitalized with either septicemia or meningitis. Of the 49, seven were newborns and 42 were adults; of the adults, all had an underlying illness or were taking immunosuppressants. This last point is in contrast to the outbreak caused by the coleslaw in which none of the nonpregnant adults had any evidence of underlying illness *(67)*.

A third outbreak was associated with consumption of a specific brand of Mexican-style white cheese *(44)*. There were 86 cases of listeriosis with 29 deaths. Almost two-thirds of the cases were among mother-newborn pairs.

Besides its ability to grow at refrigeration temperatures, *L. monocytogenes* is reported to be more

pathogenic when grown at low temperatures *(32)*. This property may have been a factor in the first two outbreaks. Holding the foods in the cold enriched for *L. monocytogenes* and, even though there were likely relatively few cells, these few cells may have been highly virulent.

ENTEROTOXIGENIC *ESCHERICHIA COLI*

Escherichia coli, a gram-negative, lactose-fermenting rod-shaped bacterium, is the most common aerobic bacterium of the large bowel of humans. It is also found in the lower intestinal tract of many warm-blooded animals. Its presence in food and water has traditionally been an indicator of fecal contamination. In pasteurized dairy products, the presence of detectable *E. coli* is indicative of either underprocessing or post-pasteurization contamination.

Until recently, the presence of the organism in foods was considered not hazardous. With outbreaks of gastroenteritis in the early 1970s in the United States traced to soft, fermented cheeses *(53),* that traditional belief was changed. It became recognized that certain strains of *E. coli* are pathogenic for both man and other warm-blooded animals, and that food can serve as a vehicle for these organisms *(52)*. These strains of *E. coli* were designated as enteropathogenic and defined as any strain of *E. coli* which can cause diarrheal disease *(47)*. These strains can be further differentiated into four groups: (a) the classical enteropathogenic strains, (b) colohemorrhagic strains, (c) enteroinvasive strains and (d) enterotoxigenic strains (designated ETEC). ETEC strains produce either a heat stable enterotoxin (ST) or a heat labile enterotoxin (LT) or both. It is the toxin(s) produced by ETEC after large numbers of the organism attach to the intestine via a colonization factor which produces the disease syndrome in man.

Kornacki and Marth *(47)* have reviewed various factors which affect enterotoxin synthesis by ETEC strains. At the time of their review, holding of food at 5°C seemed to be adequate to restrain the growth of ETEC strains and prevent toxin production by them *(47,50)*. However, recent work by Olsvik and Kapperud *(56)* has revealed that toxin-producing strains of *E. coli* were able to grow and produce ST at 4°C in both broth and broth with cream. Growth of *E. coli* at refrigeration temperature (5°C) is not completely unexpected, especially in dairy products, since Witter *(83)* had previously cited several reports about the ability of *E. coli* to grow at low temperatures. Witter *(83)* also noted that other members of the family *Enterobacteriaceae* are capable of growth at low temperature in dairy products and other foods.

The apparent, widely occurring growth of various members of the *Enterobacteriaceae* at 5°C is of further concern to food microbiologists. Sack *(64)* reported that control of toxin production (ST and LT) in ETEC is located on a plasmid and that these plasmids can be readily transferred from the toxin-producing strain to other strains of *E. coli (64)* as well as to *Salmonella typhimurium* and *Salmonella cholerasuis*. How wide ranging this ability to transfer the toxin-coding plasmid occurs is not known and whether this transfer can occur among organisms in foods is also not known. The potential exists for the growth of ST-producing strains at low temperatures by various members of the enteric group. Food microbiologists need to be aware of this potential and check for these organisms and at least consider them when investigating food poisoning outbreaks.

Hill *(37)* and Hill and Payne *(38)* have developed procedures for detecting the enterotoxigenic potential of strains of *E. coli*. These procedures are necessary because it is no longer sufficient to simply identify *E. coli* in foods.

AEROMONAS HYDROPHILA

Aeromonas hydrophila is a facultatively anaerobic, gram-negative, rod-shaped bacterium currently classified in the family *Vibrionaceae (6)*. The organism occurs widely in nature, but is especially common in water supplies. *A. hydrophila* has long been recognized as a pathogen of fish and frogs *(59)*. Early studies revealed the ability of this bacterium to grow at 1 to 5°C *(23)*. The organism is now receiving renewed interest as a human pathogen and is being isolated from cases of human diarrhea *(11,13,26,35,60)*. Buchanan *(12)* has indicated that *A. hydrophila* is one of a group of pathogens which is emerging as a foodborne organism of increasing concern. The vehicle of transmission is not currently known, although food is very likely to be involved. Studies currently in progress in this laboratory are directed toward clarifying the role of food as a vehicle for *A. hydrophila,* including defining the organism's occurrence in foods and identifying the adequacy of various tests and procedures for predicting pathogenicity of isolates recovered from foods.

A recently completed study *(58)* revealed that clinical isolates of *A. hydrophila* are capable of growth from 4 to 42°C. At 4°C, the five isolates studied in detail grew from a starting count of 10^3/ml to over 10^8/ml in 14 d. Other clinical isolates also grew well at this temperature. In a just completed study, Palumbo et al. *(57)* described a newly developed starch-ampicillin medium for the quantitative detection of *A. hydrophila* in foods. This medium provided the quantitative detection of *A. hydrophila* in all retail fish and seafood, poultry, red meat and raw milk samples surveyed. It was observed that the count per g or ml of food increased 10- to 1000-fold during 1 wk of storage at 5°C. *A. hydrophila* grew in the presence of large numbers of competing microflora in the various food products, and studies currently in progress in this laboratory indicate that even greater growth can be achieved in the absence of competing microflora and that vacuum packaging does not delay growth at 5°C.

SURVIVAL

It has been observed over the years that many organisms, including foodborne pathogens, survive better at 5°C than at room temperature or higher. For example, Christopher et al. *(19)* studied the survival of *Campylobacter jejuni* in sterile milk, beef and ground beef as a function of temperatures from -20°C to 40°C. Survival was best at 1 and 10°C. Barrell *(5)* observed longer survival of *C. jejuni* in unpasteurized milk at refrigeration temperature compared to 21°C. Over a 6-d storage period at 4°C. Hanninen *(34)* observed no change in the count of *C. jejuni* and *Campylobacter coli* in ground beef liver; the background microflora did increase over this period.

Although brucelloisis is not a current problem in the United States, the survival of *Brucella* is affected by temperature as well as other factors. Bryan *(14)*, in reviewing available literature, reported that brucellae survive longer when a food is held at low temperature (8°C) as compared to higher temperatures (25 or 37°C). Brucellae survive for long periods (up to 6 months) in Cheddar and Limburger cheeses cured at 4.4°C *(30)*.

PATHOGENIC AND INDICATOR ORGANISMS CAPABLE OF GROWTH AT TEMPERATURES SLIGHTLY ABOVE 5°C

Frequently food is temperature abused, that is, held at temperatures above the desired 5°C, hence this section will address bacteria that can grow at temperatures between 5 and 12°C. Pathogenic bacteria included in this section are: *Vibrio parahaemolyticus, Bacillus cereus, Staphylococcus aureus* and *Salmonella*. Beuchat *(9)* reported that the minimum temperature of growth of *V. parahaemolyticus* is a function of environmental factors, such as food substrate, pH and aeration. The lowest reported temperature for growth on laboratory media was 5°C and on food (oysters) was 8°C *(80)*. Goepfert et al. *(31)* reported that the lower temperature limit of growth of *B. cereus* is between 10 and 12°C. Mol *(54)* reported the spores will germinate at 8°C, but will not grow. He found that the lower limit for growth (visible turbidity in 4 to 5 d) was 12°C; no growth was observed at 8°C after 4 months. However, Chung et al. *(20)* have reported that a psychrotrophic strain of *B. cereus,* originally isolated from raw milk, grew from a vegetative inoculum at 7°C in 4 to 5 d in GYE medium. *S. aureus* grew in sterile custard at 7.7°C in 6 d *(2)*. Genigeorgis et al. *(29)* found *S. aureus* S-6 produced toxin in laboratory-cured ham at 10°C.

As with the above organisms, the minimum temperature of growth for *Salmonella* is a function of various cultural conditions as well as the food itself. Angelotti et al. *(2)* reported *Salmonella* growth in sterile chicken ala king (pH 6.2) at 6.7 to 7.7°C, but not 5.6°C. No growth was observed in ham salad (pH 5.6) or custard (pH 6.8) at 4.4 to 10°C. Matches and Liston *(51)* observed *Salmonella* growth in broth at 5.2°C after 19 d. Alford and Palumbo *(1)* observed the growth of three serotypes of *Salmonella* within 10 d at 10°C in ground pork containing 2% NaCl and normal meat spoilage microflora. In a second experiment, a mixture of the three serotypes increased in number in ground pork with 3.5% NaCl held at 10°C for 10 d.

Iandolo and Ordal *(41)* observed that heat-injured *S. aureus* will repair at 10°C. Recent studies by Smith et al. *(72)* revealed that, once heat-injured cells have repaired, the repaired cells will produce toxin. Hence, even heat-damaged cells can also create a hazard in foods if the temperature is 10°C or above.

Two other organisms, *Streptococcus faecalis* and *Streptococcus faecium,* will also be addressed even though they are generally considered not to be pathogenic. These species are termed enterococci because of their intestinal origin. Bryan *(14)* has reported that the presence of these organisms in food does not constitute a direct public health hazard. However, their presence in a food is a reflection of fecal contamination, and are valuable as indicator organisms. Because these organisms are capable of growth at 10°C and in 6.5% NaCl as well as being fairly heat resistant (survive 60°C for 30 min) *(22)*, they can grow and/or survive many food-holding conditions. Having an indicator organism capable of growth under conditions of moderate temperature abuse could partially or completely invalidate its value as an indicator organism.

CONCLUSIONS AND GENERAL CONSIDERATIONS

It was found in the mid-1950s that refrigerated holding of fresh foods slows but does not prevent the growth of food spoilage bacteria. We now know that refrigeration (5°C) cannot be relied on absolutely to keep foods safe, because some pathogens can survive and grow at this temperature. The organisms considered in this review are associated primarily with foods of animal origin, and hence, any fresh, unheated (unprocessed) food of animal origin may become hazardous with extended refrigerated storage. However, because cross-contamination can occur during handling, any fresh, refrigerated food may be suspect after extended refrigeration. The organisms considered in this review often can grow competitively in various foods.

The overall philosophy that proper refrigeration (5°C holding) will insure a safe food must be reassessed. As with spoilage organisms, 5°C holding of a food will only delay and not prevent the growth of many of the pathogens discussed in this review, and which are present in a food. These concepts should be kept in mind when formulating refrigerated products.

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